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ARMED FORCES RADIOBIOLOGY RESEARCH INST BETHESDA MD  
EFFECT OF CURARE ON RESPONSES TO DIFFERENT PUTATIVE NEUROTRANSM--ETC(U)  
JUN 76 D O CARPENTER, J W SWANN, P J YAROWSKY  
AFRRI-SR76-27

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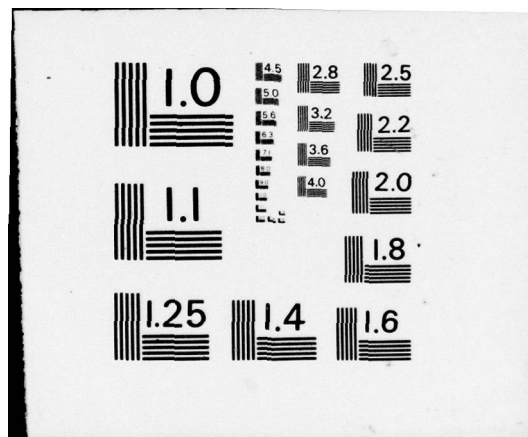
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFRRI-SR76-27	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) EFFECT OF CURARE ON RESPONSES TO DIFFER- ENT PUTATIVE NEUROTRANSMITTERS IN APLYSIA	5. TYPE OF REPORT & PERIOD COVERED	
7. AUTHOR(s) D. O. Carpenter, J. W. Swann and P. J. Yarowsky with appendix by P. R. Myers and J. A. Willis	6. CONTRACT OR GRANT NUMBER(s)	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Armed Forces Radiobiology Research Institute Defense Nuclear Agency (AFRRI) Bethesda, Maryland 20014	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NWED QAXM C 912 05	
11. CONTROLLING OFFICE NAME AND ADDRESS Director Defense Nuclear Agency (DNA) Washington, D. C. 20305	12. REPORT DATE June 1976	13. NUMBER OF PAGES 35
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Scientific rept.	15. SECURITY CLASS. (of this report) UNCLASSIFIED	
15a. DECLASSIFICATION/DOWNGRADING SCHEDULE		
16. DISTRIBUTION STATEMENT (of this Report)  Approved for public release; distribution unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Aplysia neurons have specific receptors for acetylcholine, dopamine, octop- amine, phenylethanolamine, histamine, $\gamma$ -aminobutyric acid, glutamic acid and aspartic acid. All of these substances may cause either a $\text{Na}^+$ or $\text{Cl}^-$ conductance increase response, and most may also cause a $\text{K}^+$ conductance increase response. Curare blocks all $\text{Na}^+$ and $\text{Cl}^-$ responses irrespective of which transmitter elicits the conductance change. These results indicate that any neurotransmitter, at least		

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20. ABSTRACT (continued)

in Aplysia, may be either excitatory or inhibitory. This conclusion has significance in understanding a variety of human diseases in which synthesis or metabolism of one or more transmitters is abnormal, such as Parkinson's disease, various choreas and athetoses and possibly some mental illnesses such as schizophrenia. The elucidation of the mechanisms whereby histamine affects neurons provides a basis for understanding how histamine, released from mast cells by ionizing radiation, might activate receptors on muscle of cerebral blood vessels to cause early transient incapacitation following exposures to very high doses of ionizing radiation.

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## SUMMARY

We have studied the effects of curare on responses resulting from ionophoretic application of several putative neurotransmitters onto Aplysia neurons. These neurons have specific receptors for acetylcholine (ACh), dopamine, octopamine, phenylethanolamine, histamine,  $\gamma$ -aminobutyric acid (GABA), aspartic acid, and glutamic acid. Each of these substances may on different specific neurons elicit several types of response. Fast depolarizing  $\text{Na}^+$  and hyperpolarizing  $\text{Cl}^-$  conductance increase responses have been found for all of these putative transmitters and a slow hyperpolarizing  $\text{K}^+$  conductance increase response has been found for most. All responses resulting from either  $\text{Na}^+$  or  $\text{Cl}^-$  conductance increases, irrespective of which putative transmitter activated the response, were sensitive to curare. Most were totally blocked by  $\leq 10^{-4}$  M curare. GABA responses were less sensitive and were often only depressed by  $10^{-3}$  M curare.  $\text{K}^+$  conductance responses, irrespective of the transmitter, were not curare sensitive. These results are consistent with a model of receptor organization in which one neurotransmitter receptor may be associated with any of at least three ionophores, mediating conductance increase responses to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ , respectively. In Aplysia nervous tissue, curare appears not to be a specific antagonist for the nicotinic ACh receptor but rather to be a specific blocking agent for a class of receptor-activated  $\text{Na}^+$  and  $\text{Cl}^-$  responses.

## PREFACE

We thank W. G. Shain, Jr. for many helpful discussions and G. L. Gaubatz for assistance with some of the experiments.



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## INTRODUCTION

Curare has long been considered to be the stereotypic specific antagonist to the nicotinic acetylcholine (ACh) receptor.<sup>13</sup> Curare reversibly blocks nicotinic ACh responses at low concentration.<sup>14,22</sup> Moreover, curare blocks at least part of the binding of the specific nicotinic antagonist  $\alpha$ -bungarotoxin to ACh receptors from frog neuromuscular junction<sup>30</sup> and electroplax.<sup>7</sup> Furthermore, labeled calabash alkaloids bind specifically and saturably at the neuromuscular junction.<sup>39</sup>

For Aplysia neurons the effects of curare appear to be more complex. Three kinds of response to ACh have been described, resulting from conductance increases to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ , respectively.<sup>27</sup> None of these responses is either clearly nicotinic or muscarinic, but they are pharmacologically distinguishable. The  $\text{Na}^+$  and  $\text{Cl}^-$  ACh responses are sensitive to curare, but the  $\text{K}^+$  response is sensitive to tetraethylammonium (TEA) and not curare.<sup>27</sup> In addition, the  $\text{Na}^+$  response is selectively sensitive to hexamethonium. All three responses are reversibly blocked by  $\alpha$ -bungarotoxin.<sup>37</sup>

Curare has effects on responses to putative transmitters other than ACh on Aplysia and snail neurons. Gerschenfeld<sup>18</sup> has described three conductance increase responses to serotonin, due to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ , respectively. As with ACh, the  $\text{Na}^+$  and  $\text{Cl}^-$  responses to serotonin are blocked by curare. In addition, Ascher<sup>1</sup> has demonstrated a depolarizing  $\text{Na}^+$  conductance increase response to dopamine in Aplysia neurons which is also blocked by curare. The responses due to a conductance increase to  $\text{K}^+$  elicited both by serotonin and dopamine are curare insensitive.

We have recently examined the properties of Aplysia neuron receptors to other putative neurotransmitters including histamine,<sup>5</sup> octopamine,<sup>3</sup> dopamine,<sup>38</sup> phenylethanolamine,  $\gamma$ -aminobutyric acid (GABA) and glutamic and aspartic acids<sup>40</sup> (also our unpublished observations). We have found that specific receptors for each of these substances exist and that, almost without exception, at least three different responses can be found to each substance, resulting from conductance increases to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ , respectively. Gerschenfeld and Paupardin-Tritsch<sup>19</sup> have noted that the different time courses of the  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  responses to serotonin are similar to those of the



comparable ionic responses to ACh. Swann and Carpenter<sup>38</sup> compared the properties of the three responses to ACh and dopamine and found similarities in those resulting from different transmitters but the same ionic conductance. The observations that three types of responses are found for several putative neurotransmitters, that the characteristics of the voltage change is a function of which ion moves rather than which transmitters activated the response, and evidence suggesting that the binding site for at least some of the transmitters may be identical on neurons giving  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  responses led Swann and Carpenter<sup>38</sup> to suggest a model of receptor organization. They proposed that the functional receptor unit (the receptor complex) is composed of the transmitter binding site (the receptor) and the conductance-mediating entities (ionic channels), which we will call ionophores (cf. Changeux et al.<sup>8</sup>). They suggested that the receptors for at least nine putative neurotransmitters and the ionophores mediating conductance changes to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ , respectively, are distinct and structural entities in the membranes of these neurons and can be assembled in any combination.

If this model of receptor organization is correct, it should be possible to find some pharmacologic agents that specifically block ionophores and are thus active against responses of a given conductance regardless of the transmitter. In this report, we will show that curare appears to be an antagonist not only to some ACh responses, but also to other responses which are mediated by either  $\text{Na}^+$  or  $\text{Cl}^-$  conductance increases.

Preliminary reports of some of this work have been published by Carpenter et al.<sup>6</sup> and Carpenter and Gaubatz.<sup>4</sup>

#### METHODS

Buccal, cerebral, pleural, pedal, and abdominal ganglia of Aplysia californica (obtained from Pacific Biomarine Supply Company) and Aplysia dactylomela (obtained from Marine Specimens, Unlimited) were removed from the animal, pinned to a Sylgard (Dow Company) layer in a plastic chamber<sup>35</sup> and perfused with artificial seawater (Triton Marine Salts). When appropriate, individual neurons were identified using the



criteria and nomenclature of Frazier et al.<sup>15</sup> for abdominal, Kandel and Gardiner<sup>23</sup> for buccal, and Kehoe<sup>26</sup> for the pleural cells. No major differences were found on comparison of identified cells of the two species.

Neurons were penetrated with either a double-barreled glass microelectrode or two independently inserted glass microelectrodes filled with 2 M K acetate and having resistances of 5-10 M $\Omega$ . Recordings were made through one channel utilizing a Bak impedance unity gain electrometer, a Tektronix 565 oscilloscope with 3A3 preamplifiers and a Brush Mark 200 penwriter. All measurements were made from penwriter records. The other electrode (or barrel) was used for current passage through the bridge circuit of a second electrometer. The voltage drop across the  $10^8 \Omega$  in the bridge was monitored in order to calculate the current passed.

Experiments were performed at room temperature. The reference electrode consisted of an agar-seawater bridge connected to an Ag-AgCl electrode. Electrodes were allowed to stabilize for 60-90 min before measurements were made in order to decrease changes in junction potentials. All measurements of absolute membrane potential were made upon withdrawal of the electrode at the end of the experiment.

Ionophoretic drug applications were made utilizing the control module described in detail in the Appendix. Drugs were applied through a five-barreled microelectrode, obtained from the National Institutes of Health glass shop and drawn on a vertical Kopf microelectrode puller. If necessary, the electrode tip was bumped to give a resistance of 10-20 M $\Omega$  in each barrel when filled with the appropriate putative transmitter. Electrodes were filled with distilled water by boiling and after removing as much water as possible, barrels were filled with drugs of the following concentrations: acetylcholine chloride (2 M), octopamine hydrochloride (1 M), dopamine hydrochloride (1 M), phenylethanolamine hydrochloride (1 M), histamine diphosphate (1 M),  $\gamma$ -amino-n-butyric acid (1 M), L-aspartic acid (1 M), and L-glutamic acid (1 M). All substances except glutamic and aspartic acids were ionophoresed at pH 3-4. When necessary, as with GABA, pH was adjusted by titration with concentrated HCl. Aspartic and glutamic acids were used at pH 8-9 after titration with concentrated NaOH. Curare (d-tubocurarine chloride) was obtained from Sigma, and was applied by bath perfusion.

Table 1 shows composition of the seawater solutions. Experiments were routinely performed in artificial seawater containing an added 100 mM  $\text{MgCl}_2$ . Although hyperosmotic (1300 mosmols), this solution will after a 30- to 60-min exposure effectively suppress transmitter release from presynaptic terminals.<sup>29</sup> As a consequence, it assures that a response elicited by ionophoretic drug application is a result of receptors present on that neuron and not secondary to drug effects on a second cell.

Table 1. Composition of Solutions (mM)

Components	High $\text{Mg}^{++}$ Control	$\text{Na}^{+-}$ Poor	$\text{Cl}^{-}$ - Free
$\text{NaCl}$	467	-	-
$\text{KCl}$	10	10	-
$\text{CaCl}_2$	10	10	-
$\text{MgCl}_2$	122	122	-
$\text{MgSO}_4$	29	29	29
$\text{NaHCO}_3$	3	3	3
TRIS Base + HCl to pH 7.8	-	467	-
Na - acetate	-	-	467
K - acetate	-	-	10
Ca - acetate	-	-	10
Mg - acetate	-	-	122

## RESULTS

Effects of curare on responses to ACh. Figure 1 shows the three types of conductance-increase responses to ACh which are commonly found from Aplysia neurons and the ability of curare to block these responses. The upper traces are from



Figure 1. Effects of curare on  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  conductance increase responses to ACh. The upper trace is the intracellular recording; the lower indicates the duration of the ionophoretic pulse. Part A is from neuron  $\text{R}_{15}$  which was hyperpolarized to  $-55$  mV by applying current, thus preventing spontaneous discharge. B is from neuron  $\text{R}_2$  at a resting potential of  $-48$  mV, while C is from  $\text{R}_{14}$  at  $-45$  mV. Column 1 shows control response to 300, 500 and 1000 nC ACh, respectively. Curare was applied (Column 2) at  $10^{-4}$  M,  $3 \times 10^{-5}$  M and  $10^{-3}$  M for 10, 10 and 15 min in A, B and C, respectively. After washing (Column 3) for 20, 25 and 15 min, respectively, the blockade of  $\text{Na}^+$  and  $\text{Cl}^-$  responses was reversed. Calibration: 10 mV (A and B), 5 mV (C), 10 sec.

recordings made in the neuron  $\text{R}_{15}$  (Figure 1A), in which the ACh response is known to result primarily from a conductance increase in  $\text{Na}^+$ .<sup>2</sup> This response was almost totally (Figure 1-A2) and reversibly (Figure 1-A3) blocked by a 10-min exposure to  $10^{-4}$  M curare. In four neurons with a  $\text{Na}^+$  conductance increase response to ACh, dose response curves to curare were obtained by sequential 15-min exposures to increasing concentrations. The concentrations of curare which gave a response amplitude 50



percent that of the control ( $I_{50}$ ) varied between  $3 \times 10^{-6}$  M and  $5 \times 10^{-4}$  M. In neither this or other neurons did curare ( $\leq 10^{-3}$  M) affect membrane resistance or membrane potential.

Figure 1B shows an ACh response due to a conductance increase to  $\text{Cl}^-$ , as indicated from the observations that it reverses polarity at about  $-60$  mV<sup>26</sup> and becomes depolarizing on replacement of the  $\text{Cl}^-$  of the perfusing solution with acetate. This response was totally blocked by  $3 \times 10^{-5}$  M curare. The range of  $I_{50}$  values for curare sensitivity of eight  $\text{Cl}^-$  ACh responses was similar to that of the  $\text{Na}^+$  responses. The varying sensitivity of both  $\text{Cl}^-$  and  $\text{Na}^+$  responses to curare did not appear seasonal, and was present even in successive experiments on the same identified neuron.

The records in Figure 1C show the ACh response from neuron R<sub>14</sub>. This response results from an increased  $\text{K}^+$  conductance, as indicated by a reversal potential of about  $-80$  mV, the prolonged time to peak, abolition of the response by cooling to  $8^\circ\text{C}$ , sensitivity to TEA, and insensitivity to changes in external  $\text{Cl}^-$ .<sup>26</sup> This response was insensitive to  $10^{-3}$  M curare for 15 min. We have never observed any significant depression of an ACh  $\text{K}^+$  conductance response by curare. These effects of curare on the three ACh responses are consistent with the observations of Kehoe.<sup>27</sup>

Effects of curare on dopamine responses. Three types of responses to dopamine were found which displayed many similarities to the three ACh responses.<sup>38</sup> Figure 2 shows a depolarizing dopamine response. Unlike the receptors for ACh, which are located on the somata as well as neuropile, the receptors for dopamine are found exclusively in the neuropile. This fact is probably the reason why greater ionophoretic charge was usually required to elicit a dopamine response of amplitude similar to that from ACh. Figure 2B shows that  $10^{-4}$  M curare abolished this response within 5 min. The response was also reversibly abolished by replacement of external  $\text{Na}^+$  with  $\text{Tris}^+$  (Figure 2C). These results are similar to those reported by Ascher.<sup>1</sup>

Figure 3 shows the effect of curare on a  $\text{Cl}^-$  conductance increase to dopamine. This response was abolished by a 15-min exposure to  $10^{-4}$  M curare (Figure 3B) and was identified as being due to a  $\text{Cl}^-$  conductance change since it reverses at a potential

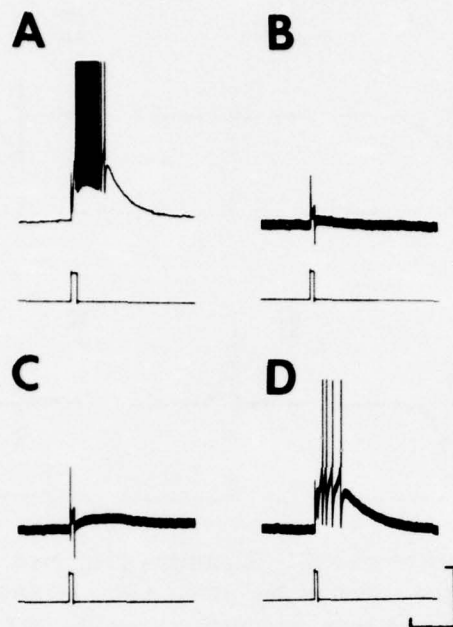


Figure 2. Blockade of a depolarizing response to dopamine by curare. Recording was made from an unidentified neuron in the lower left quadrant of the abdominal ganglion, hyperpolarized to  $-60$  mV to prevent spontaneous discharge. Dopamine pulses ( $1000$  nC) are indicated in the lower trace. A is control and B was  $5$  min after exposure to  $10^{-4}$  M curare. After recovery from curare, C shows abolition of the response after a  $5$ -min perfusion of  $\text{Tris}^+$  ( $\text{Na}^+$  free) seawater, with recovery on washing (D). Calibration:  $10$  mV,  $10$  sec.

considerably less than  $-80$  mV (Figure 3E) and is abolished by perfusion of  $\text{Cl}^-$  free (acetate) seawater (Figure 3F).

Responses to other amine putative transmitters. The effect of curare on a depolarizing response to histamine is shown in Figure 4. As with all the depolarizing responses we have studied, the histamine response was totally (Figure 4B) and reversibly (Figure 4C) blocked by curare. This response was absent in  $\text{Tris}^+$  seawater (Figure 4E), suggesting that it results from a  $\text{Na}^+$  conductance increase. The attempts to measure a conductance change during the response (Figure 4F) were unsuccessful, probably due to the fact that these histamine receptors are so far out in the neuropile that the conductance change cannot be measured in the soma. A similar suggestion

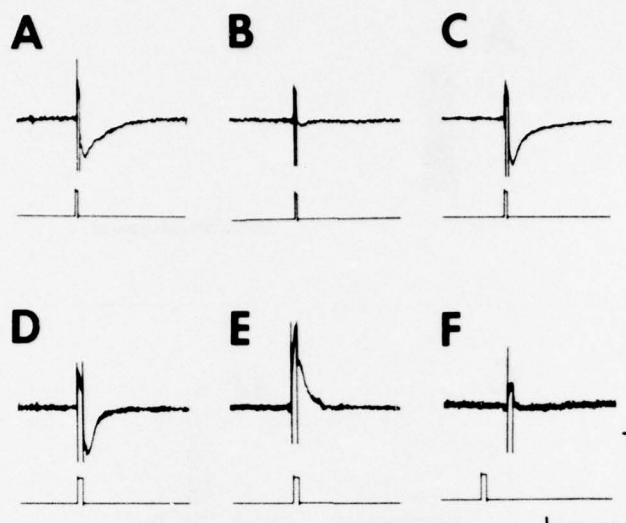


Figure 3. Effects of curare on a  $\text{Cl}^-$  dopamine response. The pulse is indicated in the lower trace and was 350 nC in A-C and 1000 nC in D-F. Recording (upper trace) was from an unidentified pleural neuron at a membrane potential of -40 mV (except for E). A shows control response, B shows the response 15 min after application of  $10^{-4}$  M curare and C shows reversal of the curare inhibition on washing with seawater. D shows control at higher ionophoretic current, while the response after hyperpolarization to -80 mV is shown in E, and F indicates abolition at -40 mV following perfusion with acetate ( $\text{Cl}^-$  free) seawater. Calibration: 10 mV, 10 sec.

has been made by Ascher<sup>1</sup> to explain the absence of measurable conductance changes on some dopamine responses. As with most of the putative transmitters we have studied, some responses to histamine showed clear conductance increases but most showed no change. In those cases where a conductance increase could be detected, the ionophoretic electrode was always relatively close to the soma.

Figure 5 illustrates an experiment on a hyperpolarizing response to phenylethanolamine. This neuron was totally insensitive to dopamine, noradrenaline, octopamine, and histamine. The response could be reversed by hyperpolarization to -73 mV (Figure 5B) even though the receptors were in the neuropile. Curare ( $10^{-4}$  M) reversibly blocked the response within 10 min (Figure 5C). The response was also abolished by perfusion with  $\text{Cl}^-$  free (acetate) seawater. Because of the short time to peak (2.5 msec) of the response,<sup>38</sup> the reversal potential at a level more depolarized than



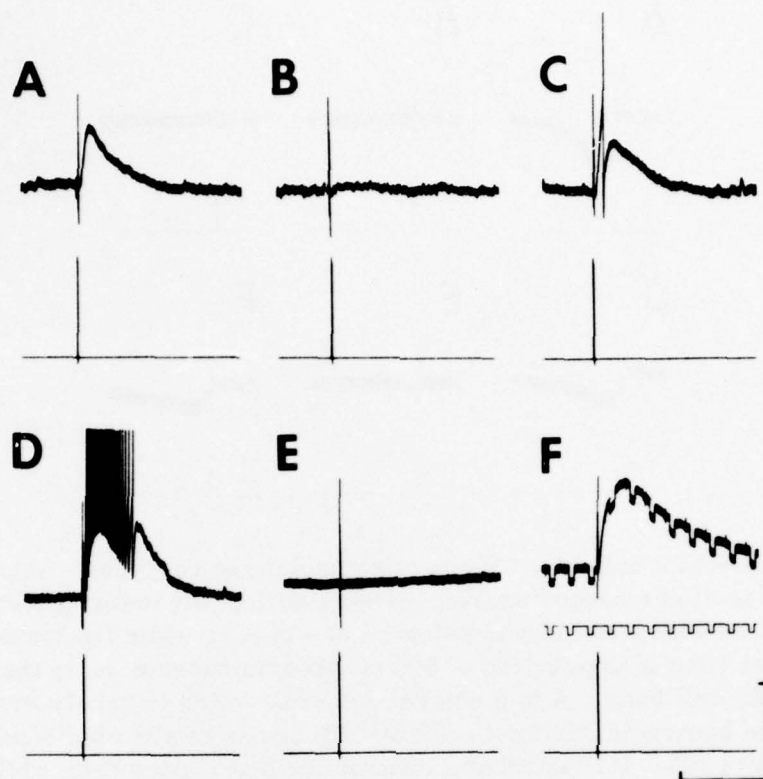


Figure 4. Curare blockade of a depolarizing histamine response. Upper trace shows recording from an unidentified neuron in the lower right quadrant of the cerebral ganglion ( $-38$  mV), while the lower trace indicates the pulse of 200 nC (A-C) or 500 nC (D-E) histamine. The current pulses in the middle trace of F are  $8 \times 10^{-10}$  A. A shows control response, B shows abolition of the response by a 5-min exposure to  $10^{-4}$  M curare and C shows recovery after a 15-min wash with seawater. The larger response in D was totally blocked after a 15-min perfusion with  $\text{Tris}^+$  ( $\text{Na}^+$  free) seawater (E). After 5 min of wash with normal seawater (F), the histamine response has recovered without return of control spike generation, and under these circumstances constant current pulses fail to demonstrate a conductance change during the response. The membrane did not show rectification over the potential spread of the response. Calibration: 10 mV, 10 sec.

$-80$  mV, and the sensitivity to removal of external  $\text{Cl}^-$ , this response can be ascribed to a specific increase in  $\text{Cl}^-$  conductance resulting from activation of a phenylethanolamine receptor. Figure 6 shows nearly total blockade of a depolarizing phenylethanolamine response by  $10^{-4}$  M curare.

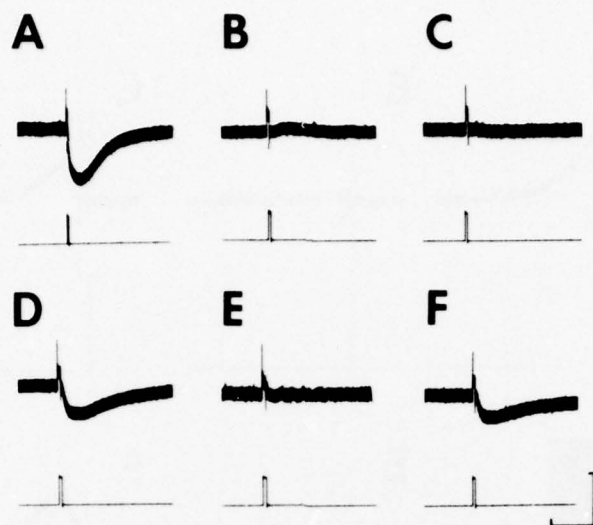


Figure 5. Curare blockade of a  $\text{Cl}^-$  phenylethanolamine response. Upper trace shows intracellular recording from an unidentified left lower quadrant cerebral neuron with a membrane potential of  $-35$  mV, while the lower trace indicates time of application of  $500$  nC phenylethanolamine to the neuropile near to the cell body. A is a control response which is barely inverted by applied hyperpolarization to  $-73$  mV (B) and is totally abolished by  $10^{-4}$  M curare (C). D illustrates a control taken at a later time while E demonstrates loss of the response in acetate ( $\text{Cl}^-$  free) seawater. F shows recovery after washing with normal seawater. Calibration:  $10$  mV,  $10$  sec.

Receptors to amino acid transmitters. Figure 7 shows the effects of curare on a GABA response. Curare ( $10^{-3}$  M for  $15$  min) depressed but did not totally abolish the response. The receptors mediating this response were located on the soma, and thus the potential was easily reversed by hyperpolarization (Figure 7E) and replacement of external  $\text{Cl}^-$  by acetate (Figure 7F). Consequently, this response appears to be a result of a specific  $\text{Cl}^-$  conductance activated by a GABA receptor. Depolarizing,  $\text{Na}^+$  conductance increase responses to GABA were also common. Both  $\text{Cl}^-$  and  $\text{Na}^+$  responses were always depressed by curare but often were not totally abolished even by  $10^{-3}$  M curare.

Figures 8 and 9 illustrate responses to aspartic and glutamic acids, respectively. For both substances,  $\text{Na}^+$  and  $\text{Cl}^-$  conductance increase responses were found frequently but  $\text{K}^+$  responses were rare for glutamic acid and have not been seen for



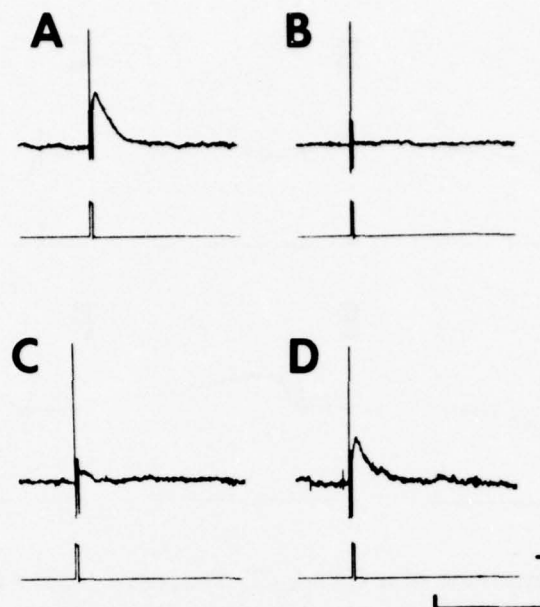


Figure 6. Effects of curare on a  $\text{Na}^+$  phenylethanolamine response (upper trace) from an unidentified buccal neuron ( $-40$  mV). Lower trace indicates times of application of  $250$  nC phenylethanolamine. This neuron was insensitive to octopamine, histamine and dopamine but was depolarized in ACh. A is a control response, B shows block in  $\text{Tris}^+$  ( $\text{Na}^+$  free) seawater. C, taken after wash and full recovery, shows blockade of the response by  $10^{-4}$  M curare for  $8$  min and D shows recovery after  $15$  min wash with seawater. Calibration:  $10$  mV,  $10$  sec.

aspartic acid. Although occasionally receptors have been found to be sensitive to both aspartate and glutamate, most receptors, as those illustrated in Figures 8 and 9, were sensitive to one but not to the other.<sup>40</sup> Curare blocked the  $\text{Na}^+$  and  $\text{Cl}^-$  responses to both aspartic and glutamic acids.

Effects of curare on biphasic responses. Kehoe<sup>26</sup> has described a two-component cholinergic response of the medial pleural neurons to ACh, consisting of an early, fast  $\text{Cl}^-$  conductance followed by a later, slower  $\text{K}^+$  conductance. We have frequently found similar biphasic responses to other putative transmitters. Figure 10 illustrates an experiment where there were biphasic responses to both ACh and octopamine. The receptors to ACh and octopamine were clearly distinct, since it was possible to desensitize one receptor by a bath application of either drug without depression of the response

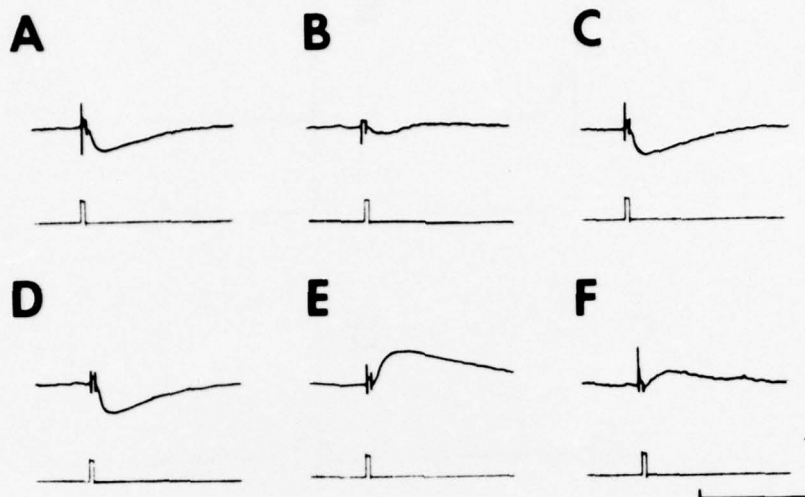


Figure 7. Curare depression of a GABA  $\text{Cl}^-$  response (upper trace) recorded from an identified neuron in the abdominal ganglion. Pulses (lower trace) were 500 nC. Potential was -50 mV except in E. The control response (A) was depressed but not totally abolished by  $10^{-3}$  M curare for 15 min (B), and recovered on washing (C). D shows a control taken at a later time at -50 mV, while E shows the inversion of the response when the cell was hyperpolarized to -66 mV. F shows reversal obtained after a 5-min perfusion with acetate ( $\text{Cl}^-$  free) seawater. Calibration: 10 mV, 10 sec.

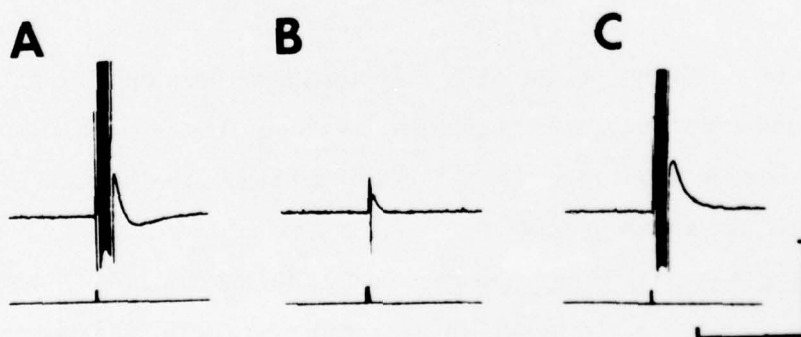


Figure 8. Effects of curare on a specific aspartate receptor from an unidentified neuron in the left lower quadrant of the abdominal ganglion. The response (A, upper trace) to 100 nC aspartate (indicated in lower trace) was almost totally blocked by  $10^{-4}$  M curare for 10 min (B) and was reversed on washing (C). Receptors were located on the neuropile and were not responsive to either glutamate or GABA. Calibration: 10 mV, 10 sec.

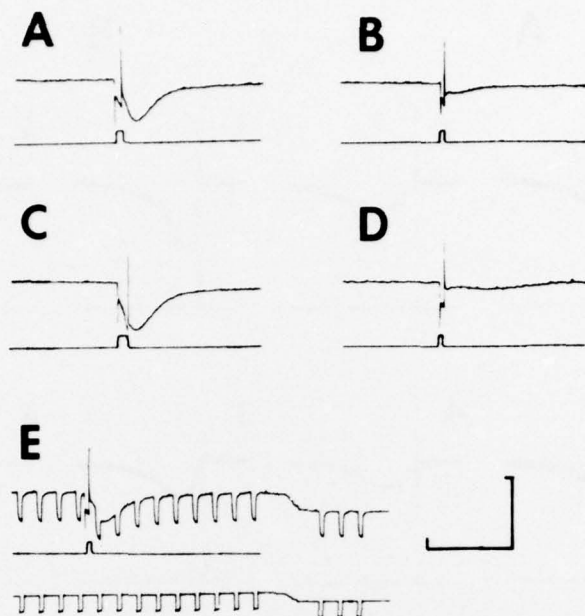


Figure 9. Blockade by curare of glutamate receptors recorded (upper trace) from an unidentified neuron in the abdominal ganglion (all records at  $-50$  mV). Control response (A) to  $500$  nC of glutamate (lower trace) is blocked by  $10^{-4}$  M curare for  $15$  min (B). A later control (C) is abolished by acetate ( $\text{Cl}^-$  free) seawater (D). E shows responses to constant current pulses ( $10^{-9}$  A). There is a clear conductance increase during the glutamate response. Hyperpolarization to a level corresponding to the peak of the response indicates that the fall in resistance is not secondary to membrane rectification. The neuron was insensitive to aspartate and GABA. Calibration:  $10$  mV,  $10$  sec.

to the other. In the control, the ACh response (Figure 10A) was biphasic even at resting potential, but when the cell was hyperpolarized to  $-72$  mV, the early component of both the ACh (Figure 10-A2) and octopamine (Figure 10-B2) responses became depolarizing, since this potential is more negative than the equilibrium potential for  $\text{Cl}^-$  ( $E_{\text{Cl}^-}$ ). After  $10^{-3}$  M curare the early component of both the ACh and the octopamine responses was abolished.

In addition to ACh and octopamine,  $\text{K}^+$  conductance increase responses have been found for dopamine, phenylethanolamine, histamine, GABA, and glutamic acid. None of these responses has been affected by curare ( $10^{-3}$  M).

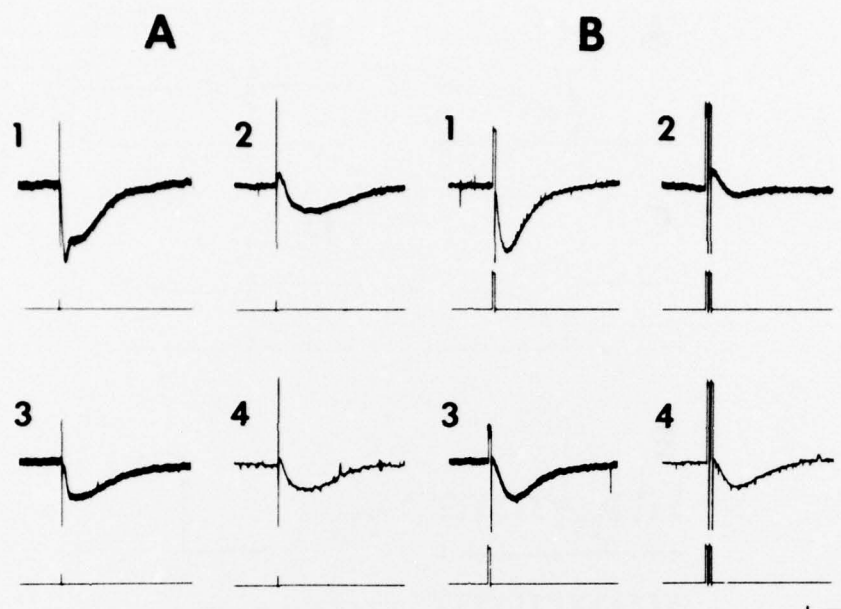


Figure 10. Effects of curare on biphasic responses to ACh (A) and octopamine (B) recorded from an unidentified lower right quadrant neuron in the abdominal ganglion. Resting potential was  $-32$  mV in A1 and 3, B1 and 3, while in A2 and 4 and B2 and 4 the neuron was hyperpolarized to  $-72$  mV. The biphasic nature of the ACh response is apparent even at resting potential (A1), but for both ACh (A2) and octopamine (B2) the early ( $\text{Cl}^-$ ) component became depolarizing after hyperpolarization. The lower records show the same responses 10 min after  $10^{-3}$  M curare and where the early component is abolished. ACh pulses were 50 nC and octopamine 1000 nC. Calibration: 5 mV, 10 sec.

#### DISCUSSION

The site of action of curare. Curare has been known to block  $\text{Na}^+$  and  $\text{Cl}^-$  responses to ACh<sup>27</sup> and serotonin<sup>19</sup> and  $\text{Na}^+$  responses to dopamine<sup>1</sup> in *Aplysia*. The results of our experiments are consistent with the generalization that  $\text{Na}^+$  and  $\text{Cl}^-$  conductance increase responses resulting from activation of specific receptors to a variety of putative neurotransmitters are curare sensitive. The only  $\text{Na}^+$  responses in *Aplysia* not curare sensitive are the slower potentials elicited from some neurons by serotonin<sup>19,20</sup> and GABA.<sup>40</sup>



Although curare effects on other transmitter actions have not been widely appreciated, Hill et al.<sup>21</sup> showed that curare blocked the inhibitory action of both GABA and glycine (presumably due to a  $\text{Cl}^-$  conductance increase) on cortical neurons of the cat. Nichol<sup>32</sup> has found that curare blocks both the depolarizing effects of GABA and  $\beta$ -alanine on primary afferents and the hyperpolarizing effects of these amino acids on spinal motoneurons. Recently, Myers et al.<sup>31</sup> have isolated a vertebrate neuronal somatic cell hybrid between a normal mouse sympathetic neuron and a neuroblastoma cell which has a depolarizing  $\text{Na}^+ - \text{K}^+$  conductance increase response to dopamine. This response is reversibly blocked by curare at very low concentrations ( $5 \times 10^{-7}$  M).

At the neuromuscular junction there is a competitive antagonism between ACh and curare (see del Castillo and Anderson<sup>13</sup>), and curare depresses miniature end-plate potentials without altering their time course.<sup>24, 25</sup> Waser<sup>39</sup> has studied binding of labeled curare and of ACh agonists to muscle. He finds differences in both quantity and distribution between ACh and curare binding sites, and suggests that curare acts by blocking the ionic channel. Since curare is effective against  $\text{Na}^+ - \text{K}^+$ ,  $\text{Na}^+$  and  $\text{Cl}^-$  conductance increase responses to several neurotransmitters, its action is most easily explained by postulating that it interferes with the conductance change rather than the receptor.

Interaction between curare and  $\alpha$ -bungarotoxin binding sites. Purified proteins from snake venoms, such as cobra toxin and  $\alpha$ -bungarotoxin, have recently been shown to be specific antagonists for the nicotinic ACh receptor.<sup>7, 33</sup> Radiolabeled toxins have been shown to bind essentially irreversibly to ACh receptors from a variety of tissues. Curare will at least partially prevent toxin binding at the neuromuscular junction<sup>30</sup> and electroplax.<sup>34</sup>

Fulpius et al.<sup>16, 17</sup> have studied interactions between labeled cobra toxin and the ACh receptor and have found a single class of toxin binding sites with a  $K_D = 2 \times 10^{-9}$ . They have then applied the kinetic analysis developed by Cleland<sup>9, 10</sup> for enzymes to the interaction of cobra toxin with ACh agonists and antagonists. They found that drugs such as carbamylcholine, hexamethonium and decamethonium gave a linear competitive inhibition of toxin binding, but that curare and several other nicotinic antagonists gave

a hyperbolic competitive inhibition. According to Cleland, a linear competitive inhibition indicates that there cannot be simultaneous binding of the interacting substances (i.e., carbamylcholine and cobra toxin). Hyperbolic competitive inhibition, however, implies that there are separate but nearby binding sites for the interacting substances (i.e., cobra toxin and curare).

$\alpha$ -Bungarotoxin is very effective in blocking responses to ACh on Aplysia neurons. Furthermore,  $^{125}\text{I}$   $\alpha$ -bungarotoxin binds to homogenates of Aplysia nervous tissue with a  $K_D = 0.8 \times 10^{-9}$  M, and greater than 90 percent of the toxin binding is prevented by curare.<sup>37</sup> We have recently applied a Cleland analysis to the interactions of carbamylcholine and curare with  $^{125}\text{I}$   $\alpha$ -bungarotoxin and confirm for the Aplysia receptor that the interaction between carbamylcholine and  $\alpha$ -bungarotoxin shows a linear competitive inhibition whereas the interaction between curare and  $\alpha$ -bungarotoxin is a hyperbolic competitive inhibition (Shain et al., unpublished). These results are consistent with the hypothesis that curare binds at a site distinct from but intimately associated with the ACh binding site.

The organization of receptors and ionophores on Aplysia neurons. Swann and Carpenter<sup>38</sup> have suggested a model of organization of neurotransmitter receptor complexes in which the receptor (transmitter binding site) and ionophore mediating the conductance change are distinct entities. On the basis of observations on Aplysia neurons, they proposed that (1) a single receptor may be associated with any of at least three ionophores which mediate permeability changes to  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$ , respectively, and (2) the ionophores are functional units which may be associated with receptors for different neurotransmitters. The effects of curare on responses to these several putative neurotransmitters provide support for this model.

A major test of this hypothesis is the search for three responses to each substance for which a specific receptor is known. In addition to ACh and dopamine,  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  responses have been found for phenylethanolamine, octopamine, histamine, GABA and glutamic acid<sup>4, 40</sup> (also Swann and Carpenter, unpublished). For aspartic acid only  $\text{Na}^+$  and  $\text{Cl}^-$  responses have been found.<sup>40</sup> These observations complement the studies of Gerschenfeld and his collaborators (summarized by Gerschenfeld<sup>18</sup>) who

have shown three responses for serotonin and glutamic acid, and in the case of serotonin, have even found several additional types of responses.<sup>19,20</sup>

If one receptor can mediate three different ionic responses, agonists which are structurally similar to the transmitter should show the same relative effectiveness in eliciting the three responses. This is the case for dopamine responses, which are also somewhat sensitive to noradrenaline and adrenaline, but not sensitive to octopamine or phenylethanolamine.<sup>38</sup> The three ACh responses are activated with similar effectiveness relative to that of ACh by choline, carbamylcholine and acetyl- $\beta$ -methylcholine<sup>27</sup> (also Niesen and Carpenter, unpublished). However, some agonists which differ considerably in structure from ACh, such as nicotine or arecoline, will selectively activate one or two but not all ACh ionic responses.<sup>27</sup> It is possible that under such circumstances the activation of the receptor complex is either not mediated by binding at the ACh receptor site or that steric differences associated with utilization of different ionophores determine the specificity.

Shain et al.<sup>37</sup> suggested that there was only one ACh receptor in *Aplysia* on the basis of experiments with  $\alpha$ -bungarotoxin. They found that  $\alpha$ -bungarotoxin blocks all three ACh responses and that labeled toxin binds to membrane homogenates with what appears to be single association and dissociation constants. Further support for a single ACh receptor has come from studies on  $\alpha$ -bungarotoxin binding to single identified neurons with known ACh responses.<sup>36</sup> The  $K_m$  for toxin binding was found to be identical in neurons which had either  $\text{Na}^+$ ,  $\text{Cl}^-$  or  $\text{K}^+$  responses to ACh.

Swann and Carpenter<sup>38</sup> compared responses due to the same ionic conductance but activated by ACh or dopamine. They found the temporal characteristics and temperature sensitivities of the responses to be a function of the ionic conductance rather than the transmitter, in agreement with previous observations.<sup>1,18,26,27</sup> The ionic selectivity of the ionophore mediating the  $\text{K}^+$  ACh response<sup>26</sup> appears to be identical to that mediating the dopamine  $\text{K}^+$  response.<sup>1</sup> These observations and the effect of curare on  $\text{Na}^+$  and  $\text{Cl}^-$  responses to nine putative neurotransmitters support the postulate that the ionophores are distinct entities which determine the characteristics of the



conductance change, which can be associated with several different receptors and can be blocked by specific pharmacologic agents.

Our studies do not explain how curare can act to block both  $\text{Na}^+$  and  $\text{Cl}^-$  responses. These ions are of opposite charge and thus the channels through which they flow should be quite different.

Other drugs appear to be specific blocking agents for one ionic response for one transmitter (such as hexamethonium on the  $\text{Na}^+$  ACh response or ergometrine on the  $\text{K}^+$  dopamine response). This is not necessarily incompatible with the model of receptor organization, since the specificity might result from steric differences among the various receptor-ionophore combinations which restrict drug sensitivity. Alternatively, there may be drug binding sites on a receptor complex which do not interfere with all three ionic responses. This appears to be the case for hexamethonium, which blocks only the  $\text{Na}^+$  ACh response but which will block  $\alpha$ -bungarotoxin binding to  $\text{Cl}^-$  and  $\text{K}^+$  ACh neurons as effectively as to  $\text{Na}^+$  neurons.<sup>36</sup>



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## APPENDIX

Ionophoretic control unit for use with multibarreled pipettes. The amount of the ionized form of a molecule expelled from a microelectrode by an applied electric field is a function of both ionophoresis and electroosmosis. The ionophoretic component may be expressed by the following equation:<sup>11</sup>

$$M_i = \frac{Ni}{ZF} \quad (1)$$

where  $M_i$  = ionic flux in moles/sec,  $i$  = current,  $F$  = Faraday's constant,  $Z$  is the magnitude of ionic charge and  $N$  = the transport number of the ion under consideration. The electroosmotic component relates to bulk flow of solvent from the microelectrode and is related to the strength of the electric field and electrode geometry as follows:

$$M_O = \frac{\pi r^2 CE}{1 \times 10^3} \quad (2)$$

where  $M_O$  = electroosmotic motility in moles/sec,  $r$  = electrode tip radius,  $l$  = electrode tip length,  $C$  = concentration of the molecule under consideration in moles/liter and  $E$  = electric field in volts.

The total electrophoretic flux ( $M_T$ ) may be expressed as the sum of the ionophoretic and electroosmotic fluxes:

$$M_T = M_i + M_O \quad (3)$$

Curtis et al.<sup>12</sup> have shown that, for solutions of concentrated electrolytes,  $M_O$  represents a small percentage of  $M_T$ . For 3 M ACh, Krnjević et al.<sup>28</sup> found that  $M_O$  was 0.11  $M_T$ . Since  $M_O$  is a function of electric potential and electrode geometry it should be independent of the molecular species, provided that the applied potential remains constant.

For electrodes filled with well ionized substances the greatest part of the electrophoretic flux will be due to ionophoresis (equation (1)). Neglecting the contribution of electroosmosis, the total quantity of ionized species ( $X_T$ ) passed in a given interval of time will be:

$$X_T = \int_0^T \frac{Ni}{ZF} dt = \frac{N}{ZF} \int_0^T i dt \quad (4)$$

We desired to be able to apply not only variable and reproducible amounts of one drug but also known relative amounts of substances very similar in structure and charge to the first drug (which can therefore be assumed to have similar transport numbers), but which are passed from different barrels of the ionophoretic electrode. A primary consideration is the electrode impedance, which is different for different electrodes and which varies in a single electrode during an experiment. If the electrode is driven by a voltage source, the amount of ions delivered will vary as the inverse of the electrode impedance, which varies and is different for different barrels. If the electrode is driven by a current source, a reproducible ionophoretic charge is delivered. However, as electrode impedance varies, the applied potential,  $E$ , varies to maintain the constant current. This causes a change in the relative electroosmotic flux, which is a function of  $E$ .

In order to minimize changes in electroosmotic flux, we have chosen to maintain a relatively constant potential and to integrate the current passed through the electrode in order to maintain a constant total ionophoretic flux. Figure A-1 is a simplified schematic diagram of the unit used. It contains a variable source for both backing and ionophoretic currents and circuitry for initiating, sensing, integrating and terminating the ionophoretic current when the preset total charge has been passed. The polarity of currents is reversible to allow for ionophoresis of either anionic or cationic species. The stimulator has two modes (run and stop) as selected by the flip-flop. When in the stopped state the unit selects the backing source and resets the integrator. When a run state is entered by manually setting the run flip-flop, the ionophoretic source is selected and the integrator (A3) begins integrating the ionophoretic current. The unit returns to the stopped state when the output of the integrator reaches a voltage corresponding to the amount of charge predetermined by the setting of the charge control.

By using digital logic, relays and several backing sources, this unit has been expanded to allow use with a multiple barreled ionophoretic electrode. The present system can deliver from 0-1000 nC at rates of from 0-1000 nC/sec from five separate channels.



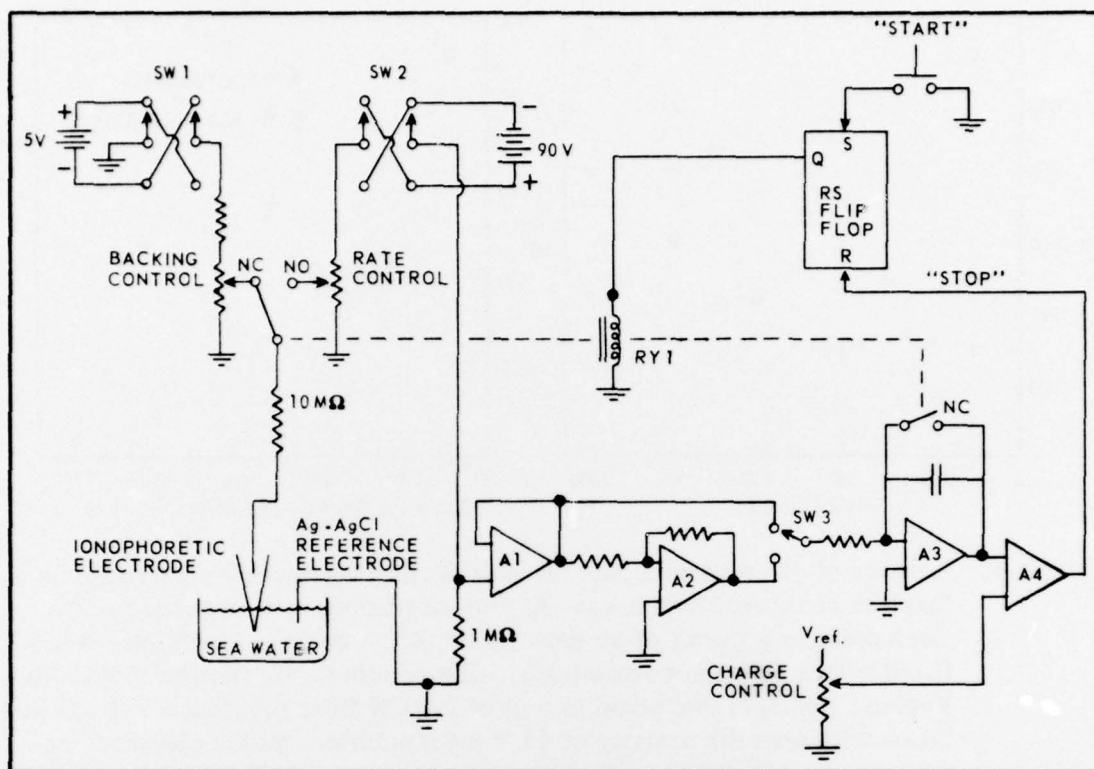


Figure A-1. Simplified circuit diagram for ionophoretic stimulator. When start is depressed the reset-set (RS) flip-flop closes relay 1. Ionophoretic current is sensed by A1 and integrated by A3. When the integrated charge reaches a predetermined amount, comparator A4 resets the RS flip-flop, opening reed relay RY 1 and terminating the ionophoretic current. In the stopped state relay 1 selects backing current. Inverter A2 and switch 3 (SW 3) allow the integrator to work both for positive and negative ionophoretic currents. Switches SW 1 and SW 2 select polarity of ionophoretic and backing currents. NC denotes "normally closed" and NO denotes "normally open."

Figure A-2(A) illustrates that the quantity of charged species passed is a linear function of the charge setting when tested using  $^3\text{H}$ -acetylcholine and  $^3\text{H}$ -dopamine. Figure A-2(B) shows released radioactivity at a constant charge setting but with increasing rate of delivery. The amount of  $^3\text{H}$ -acetylcholine released increased linearly with rate of delivery, and was 25 percent greater at 1000 than at 200 nC/sec. This increase presumably reflects that predicted by equation (2) for electroosmotic flux as the driving potential is increased.

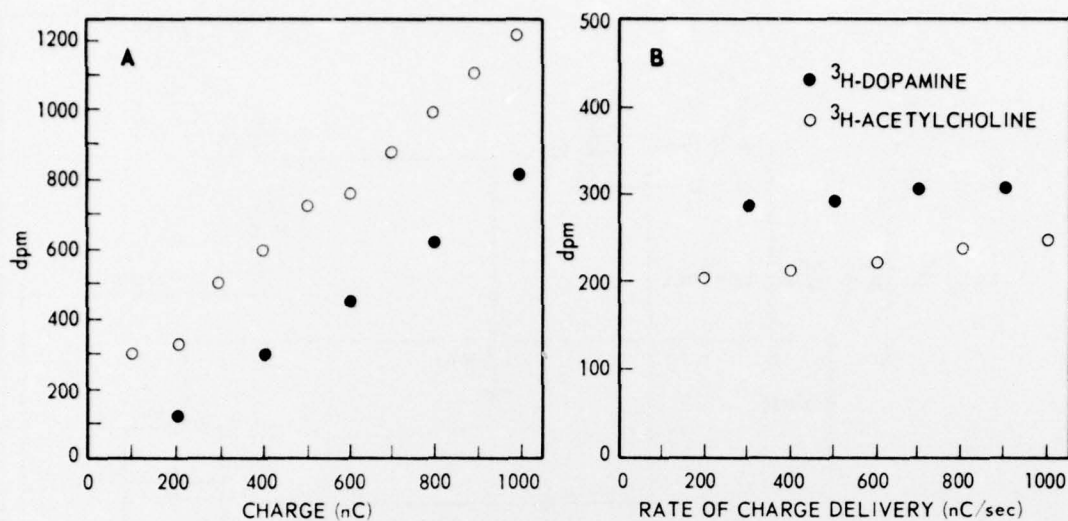


Figure A-2. Release of  $^3\text{H}$ -dopamine and  $^3\text{H}$ -ACh from ionophoretic electrodes as a function of increasing charge (A) and increasing rate of delivery (B). Each point is a result of 20 repetitions of the pulse. Electrodes were filled with a solution containing 1.6 mg of acetyl- $^3\text{H}$ -choline iodide (New England Nuclear) dissolved in 5  $\mu\text{l}$  of 0.01 N HCl, yielding a 1.17 M solution with specific activity of 49.9 mCi/mmole. Initial electrode resistance was 13.2  $\text{M}\Omega$ .  $^3\text{H}$ -dopamine was prepared by evaporating 500  $\mu\text{l}$  of 3,4-dihydroxyphenylethylamine tartrate (ethyl-2- $^3\text{H}$  (N); specific activity: 10 Ci/mmole; New England Nuclear) to dryness and redissolving the residue in 5  $\mu\text{l}$  of 1 M dopamine HCl (Sigma, made up in 0.01 N HCl). Final specific activity was 9.94 mCi/mmole. The purity of the  $^3\text{H}$ -dopamine was greater than 95 percent when compared to authentic dopamine by thin-layer chromatography. Initial electrode resistance was 14.0  $\text{M}\Omega$ .

Operation of the charge integrator was verified by simulating an ionophoretic electrode with resistors. The discharge time and current was measured for resistors from 20-220  $\text{M}\Omega$ . The current-time product over this resistance range varied less than 1 percent. A second series of tests were done with resistors placed in series with a  $^3\text{H}$ -acetylcholine electrode. Over a range of 20-220  $\text{M}\Omega$  series resistance the total transmitter delivered decreased linearly 2-3 percent. This decrease is expected, due to a decrease in the electroosmotic ejection secondary to increasing voltage drop across the series resistor and thus a decrease in potential across the ionophoretic electrode.